Molecular cloning and deduced amino acid sequences of the α - and β -subunits of mammalian NAD⁺-isocitrate dehydrogenase

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A 153 bp fragment of the cDNA encoding the β -subunit of pig heart NAD⁺-isocitrate dehydrogenase (NAD⁺-ICDH) was specifically amplified by PCR, using redundant oligonucleotide primers based on partial peptide sequence data [Huang and Colman (1990) *Biochemistry* 29, 8266–8273]. This PCR fragment was then used as a probe to isolate cDNA clones encoding the complete mature form of the β -subunit from a monkey testis cDNA library. Examination of the deduced amino acid sequence of the monkey subunit and the partial sequence of the pig heart enzyme revealed a high level of sequence conservation. In addition, 3 overlapping fragments of the cDNA for the α -subunit

of monkey NAD⁺-ICDH were amplified using oligonucleotide primers derived from the cDNA sequence of a subunit of bovine NAD⁺-ICDH (EMBL accession no: U07980). These cDNA fragments allow deduction of the amino acid sequence of the α -subunit. Since the γ -subunit of monkey NAD⁺-ICDH has already been cloned [Nichols, Hall, Perry and Denton (1993) *Biochem. J.* 295, 347–350], a deduced amino acid sequence is now available for all three subunits of mammalian NAD⁺-ICDH. Interrelationships between these subunits are discussed and they are compared with the two subunits of yeast NAD⁺-ICDH and *Escherichia coli* NADP⁺-ICDH.

INTRODUCTION

NAD⁺-isocitrate dehydrogenase (NAD⁺-ICDH) catalyses the oxidation of *threo*-D₂-isocitrate to oxoglutarate and carbon dioxide. It is located exclusively within mitochondria and is a component of the citrate cycle. Two other enzymes catalyse the same reaction in eukaryotic cells; the cytoplasmic and mitochondrial forms of NADP⁺-isocitrate dehydrogenase (NADP⁺-ICDH). Although mitochondria generally contain NADP⁺-ICDH activity greatly in excess of that of NAD⁺-ICDH, only the NAD⁺-dependent enzyme is a significant source of reducing equivalents for the respiratory chain [1–3]. NAD⁺-ICDH from pig and ox heart (and presumably other mammalian sources) is composed of three polypeptide subunits, α , β and γ . All three subunits have a molecular mass of approximately 40 kDa, as determined by SDS/PAGE. They are found in the ratio 2:1:1 respectively, and $\alpha_2\beta\gamma$ tetramers are thought to function *in vivo* as oligomeric assemblies [4,5].

NAD⁺-ICDH has complex regulatory properties which are consistent with a role in the control of citrate cycle flux. Currently, there is no indication that there are tissue specific isoforms of NAD⁺-ICDH, or that the properties of the enzyme are significantly different in various mammalian species. NAD⁺-ICDH exhibits co-operative kinetics with respect to isocitrate and is activated by increasing ADP/ATP ratios, via a decrease in the $K_{0.5}$ for isocitrate [1,6,7]. Furthermore the mammalian enzyme, but not that from invertebrates, plants or yeast, is activated by Ca²⁺ [8–10]. Pyruvate dehydrogenase phosphatase and oxoglutarate dehydrogenase from mammalian cells are also activated by Ca²⁺ [11,12]. Increases in cytoplasmic Ca²⁺, such as those caused by hormones or other extracellular stimuli, induce increases in mitochondrial Ca²⁺ which are sufficient to stimulate all three dehydrogenases [13]. Concomitant increases in mitochondrial Ca²⁺ and NADH production can be observed in living cells [14]. Thus activation of these three enzymes is an important

means whereby ATP production matches increased demand due to Ca²⁺-induced changes in cellular processes (such as proliferation, secretion and muscle contraction). Binding studies suggest that there is only one Ca²⁺-binding site per $\alpha_2\beta\gamma$ unit of NAD⁺-ICDH [7]. However, the molecular basis for the effect of Ca²⁺ on NAD⁺-ICDH is unknown, as is the contribution of each subunit to any of the catalytic and regulatory properties of the holoenzyme. The γ -subunit of mammalian NAD⁺-ICDH has previously been cloned [15]. In addition, an undocumented cDNA sequence for one of the subunits of bovine NAD⁺-ICDH has been placed in the EMBL database (accession number U07980). Although this sequence is not assigned to the α -, β - or γ -subunits, it includes areas corresponding to reported peptides from the α -subunit of pig NAD⁺-ICDH [16]. Consequently it appeared likely that this cDNA is for the bovine α -subunit. Cloning of the equivalent cDNA from monkey provides a way of reinforcing the identification of this subunit and, more importantly, allows comparison of all three subunits of mammalian NAD⁺-ICDH from a single species.

NAD⁺-ICDH from the mitochondria of the yeast *Saccharomyces cerevisiae* has two similar subunits, with a molecular mass of approximately 40 kDa. cDNAs for both of these subunits have been cloned and sequenced [17,18]. Deduced amino acid sequences are also available for both the mitochondrial and cytoplasmic isoforms of NADP⁺-ICDH from mammalian and yeast cells [19–21]. The isocitrate dehydrogenase found in *Escherichia coli* is NADP⁺-dependent, but its primary structure is more similar to eukaryotic NAD⁺-ICDHs than NADP⁺-ICDHs [22]. Several high-resolution crystal structures are available for the *E. coli* enzyme [23].

In this paper we report the cloning and deduced amino acid sequences of the α - and β -subunits of NAD⁺-ICDH from monkey testis. Complete sequences are now available for every subunit of all three ICDH isoenzymes, from both yeast and mammalian sources. Detailed comparison of the sequences and regulatory

Abbreviation used: ICDH, isocitrate dehydrogenase.

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The nucleotide sequence of monkey (*Macaca fascicularis*) NAD⁺-ICDH α -subunit and β -subunit cDNA will appear in the EMBL Nucleotide Sequence Database under the accession numbers X87172 and X82632 respectively.

properties of mammalian NAD⁺-ICDH with those of the yeast enzyme, and identification of conserved, catalytically important residues using the *E. coli* NADP⁺-ICDH structure are now possible.

MATERIALS AND METHODS

Materials

Restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, avian myeloblastosis virus reverse transcriptase and deoxynucleotides were from Pharmacia, Milton Keynes, U.K. pCR cloning vector was from InVitrogen, Leek, The Netherlands. *Taq* polymerase was from Boehringer-Mannheim, Lewes, E. Sussex, U.K. Random hexamer DNA labelling kit was from Du Pont-NEN, Stevenage, Herts., U.K. Nitrocellulose filters and microdialysis membranes were from Schleicher and Schull and Millipore respectively. All other chemicals were of AnalaR grade or the purest grade available. Construction and storage of rat (*Rattus norvegicus*) epididymal and monkey (*Macaca fascicularis*) testis cDNA libraries have been described previously [24].

PCR amplification of pig heart NAD⁺-ICDH β cDNA

Total pig heart RNA (3 μ g) was used in oligo(dT)₁₂₋₁₈-primed cDNA synthesis catalysed by avian myeloblastosis virus reverse transcriptase. PCR amplification of a portion of the cDNA for NAD⁺-ICDH β was then carried out using degenerate oligonucleotide primers based on available peptide sequence for NAD⁺-ICDH β [16]. In total, 10 primers with redundancies

ranging from 64- to 1026-fold and lengths of 17 to 20 bases were used. The relative orientation of members of each primer pair was based on alignment of pig NAD⁺-ICDH β peptide sequence with deduced amino acid sequence for both subunits of yeast NAD⁺-ICDH and of monkey NAD⁺-ICDH γ . Initial PCR experiments using previously successful reaction conditions [15] failed to amplify the desired cDNA. Consequently, reaction conditions were changed to those used by Walker et al. [25]; 5% of the cDNA preparation in 10 mM Tris/HCl, pH 8.9, containing 50 mM KCl, 1.5 mM KCl, 100 mg/ml gelatin, 0.2 mM dNTPs, 1 unit of *Taq* polymerase, and the appropriate primers was employed in a two-stage amplification. For the first 30 cycles, the concentration of each primer was 50 nM, and then for a second 30 cycles primer concentration was increased to 2 μ M. Where possible the second stage was carried out with nested primers (i.e. primers derived from peptide sequence internal to that used to design the primers for the first 30 cycles). In both stages each thermal cycle consisted of: 94 °C for 90 s; cool to 45 °C; 45 °C for 90 s; rapidly heat to 72 °C and hold for 2.5 min; rapidly heat to 94 °C. Only one combination of primers produced a band of the expected size (153 bp) when the PCR products were analysed on a 1.5% agarose gel. The peptide sequence used to design these primers and the orientation of the primers are shown in Figure 1. The sequences of the primers were 1: AAYATHGCN-AAYCCNACNGC, 2: GCTTCNGTRAARTCYTGRC and 3: GTNGCRTANCCNCCCATRTC. Primers 1 and 2 were used in the first stage amplification and primers 1 and 3 in the second stage. The PCR product was cloned into plasmid pCR and subjected to DNA sequence analysis.

Figure 1 Nucleotide and deduced amino acid sequence of NAD⁺-ICDH β from monkey testis

Also shown are the locations of peptides from pig NAD⁺-ICDH β [16]. These are generally identical to the equivalent areas in the monkey subunit, and such residues in the monkey sequence are italicized. Where there are differences between the monkey and pig sequences, the pig residue is given beneath the monkey one. The areas of cDNA sequence corresponding to PCR primers 1, 2 and 3 are identified by a numbered horizontal bar.

Figure 2 Nucleotide and deduced amino acid sequence of NAD⁺-ICDH α from monkey testis

Also shown are the locations of peptides from pig NAD⁺-ICDH α [16]. These are generally identical to the equivalent areas in the monkey subunit, and such residues in the monkey sequence are italicized. Where there are differences between the monkey and pig sequences, the pig residue is given beneath the monkey one. The areas of cDNA sequence corresponding to PCR primers A, B, C, D, E and F are identified by a labelled horizontal bar. The pairing of these primers is described in the appropriate Materials and methods subsection. The last 22 bases of the monkey NAD⁺-ICDH α cDNA sequence are unconfirmed, as this region corresponds to PCR primer F which was based on the bovine homologue.

PCR amplification of monkey testis NAD⁺-ICDH α cDNA

Monkey testis mRNA (2 μ g) was used in oligo(dT)₁₂₋₁₈-primed cDNA synthesis catalysed by avian myeloblastosis virus reverse transcriptase. PCR amplification of three overlapping portions of the cDNA for NAD⁺-ICDH α was carried out using oligonucleotide primers based on the undocumented cDNA sequence of a putative subunit of bovine NAD⁺-ICDH (EMBL database accession no: U07980). The sequences of the primers were A: TCCAAAAACAGGTGACIAGIGG, B: TCGATTGAGACACATGGTCGGACAYTT, C: CCCAGAAATTTCTGCTGCA-GTTATGAA, D: TTTACAGTTTTCTGCAACTTCCCT-YCA, E: CAGCCGTGCACAAAGCCAAC and F: CTAAA-TCTTTTACICGICGACA. Primers A and B, C and D, and E and F were paired together in three separate PCR reactions. The location of these primers in the contiguous α -subunit cDNA sequence is shown in Figure 2. Inosine residues were included at appropriate positions towards the 3'-end of primers A and F to allow for species differences in codon usage. PCR conditions were as described above. PCR products were cloned in the plasmid pCR prior to sequencing.

Screening of cDNA libraries and DNA sequence determination

Approximately 3×10^5 monkey testicular cDNA clones were transferred to nitrocellulose filters and screened for those hybridizing to the above 153 bp pig ICDH β -subunit PCR product, labelled by random priming with [³²P]dCTP. Hybridization was for 12 h at 58 °C in 0.9 M NaCl/0.09 M sodium citrate, pH 8.0. After secondary and tertiary clone purification under the same conditions, plasmid DNAs from strongly hybridizing clones were isolated and their cDNA sequences determined. All DNA sequencing was carried out on

both DNA strands using a custom primer walking strategy and employing a DuPont Genesis 2000 automated sequencer. Each NAD⁺-ICDH α PCR product sequence was produced by sequencing clones derived from at least two independent PCR reactions.

RESULTS AND DISCUSSION

Cloning and sequence analysis of cDNAs encoding the β -subunit of pig and monkey NAD⁺-ICDH

Ten degenerate oligonucleotide primers were designed using partial peptide sequence from pig heart NAD⁺-ICDH β [16] for PCR amplification of cDNA. Despite considerable effort spent in optimizing PCR parameters, only one primer combination was successful, yielding the expected 153 bp PCR product. The amino acid sequences on which the successful primers were based are shown in Figure 1.

There are some discrepancies between the deduced amino acid sequence of the 153 bp PCR product and the sequence of one of the pig β -subunit peptide fragments (Figure 1, discussed below). However, there is strong similarity between the sequence of the PCR product, that of the NAD⁺-ICDH γ -subunit and both subunits of yeast NAD⁺-ICDH. Although the above PCR product was obtained from pig RNA, the availability of a monkey cDNA library of proven quality [24] led us to screen this for the corresponding NAD⁺-ICDH β homologue. After screening approximately 3×10^5 monkey testicular cDNA clones, five strongly hybridizing clones were identified, purified and subjected to automated sequence analysis of their DNA inserts.

The resulting cDNA sequence, compiled from two large, overlapping clones, and the amino acid sequence deduced from it, are shown in Figure 1. The deduced amino acid sequence

Figure 3 Comparison of N-terminal peptide sequences of the α -, β - and γ -subunits of pig NAD⁺-ICDH [16] with the equivalent areas of monkey NAD⁺-ICDH deduced amino acid sequence [15]

Identical residues are indicated with a vertical line. The peptides are aligned for maximal identity. The published N-terminal peptide sequences for the subunits of pig NAD⁺-ICDH have been independently confirmed in our laboratory (M. J. Leake and R. M. Denton, unpublished work).

contains an area identical to that encoded by the 153 bp PCR product, and is consistent with all but one of the other reported pig NAD⁺-ICDH β peptide sequences (see below) [16]. These peptide sequences are identical to the deduced amino acid sequence of the β -subunit at 70 of the 75 available residues (Figure 1). The identity of the monkey β -subunit is further confirmed by comparison of N-terminal sequences of all three subunits of NAD⁺-ICDH, which clearly shows that N-terminal peptide sequence from pig NAD⁺-ICDH β is very similar to the corresponding area of the monkey β -subunit, but very different to that of the other subunits of NAD⁺-ICDH (Figure 3). The published N-terminal peptide sequences for the subunits of pig NAD⁺-ICDH [16] have been confirmed in our laboratory (M. J. Leake and R. M. Denton, unpublished work).

The one discrepancy is a peptide (corresponding to residues 284–303 of the β -subunit; see Figure 1), which was originally assigned by Huang and Colman [16] to both β - and γ -subunits. Our results show that it is in fact only present in the γ -subunit (see Figure 5).

The cDNA sequence of NAD⁺-ICDH β contains a putative polyadenylation/cleavage signal at its 3' end (nucleotides 1176–1181) and an open reading frame extending from nucleotides 1 to 1144. The absence of a putative initiation codon upstream of the region corresponding to the N-terminal peptide sequence from the mature β -subunit of pig NAD⁺-ICDH (Figure 1) indicates that the cDNA is truncated at its 5' end, within the presumptive mitochondrial import sequence. The identification of the N-terminus of the monkey intramitochondrial β -subunit is based on identity with the pig homologue (Figure 3). In addition, it is consistent with the general observation that mitochondrial import peptides are commonly cleaved two or three residues to the C-terminal side of basic residues, a configuration which is found at the putative cleavage site in monkey NAD⁺-ICDH β . Thus the monkey NAD⁺-ICDH β cDNA is full length with respect to the mature polypeptide.

Cloning and sequence analysis of cDNAs encoding the α -subunit of monkey NAD⁺-ICDH

Contiguous cDNA sequence for the α -subunit of monkey NAD⁺-ICDH, derived from three PCR products, is shown in Figure 2. This sequence is 96 % identical to that of the bovine subunit used in primer design. Both the sequence presented here and the bovine sequence contain all of the available pig NAD⁺-ICDH α peptide sequences [16], with identical residues at 83 of the 89 available positions. The six differences are explicable in terms of

species difference (Figure 2). As before, subunit assignment is confirmed by comparison of N-terminal sequences of all three subunits of NAD⁺-ICDH, which clearly shows that N-terminal peptide sequence from pig NAD⁺-ICDH α is almost identical to the corresponding area of the monkey α -subunit, with relatively little similarity to the β - and γ -subunits of NAD⁺-ICDH (Figure 3). Thus the monkey sequence presented here and the undocumented bovine subunit cDNA code for the α -subunit of NAD⁺-ICDH. Both of these sequences lack a putative initiation codon upstream of the region corresponding to N-terminal peptide sequence from the mature α -subunit of pig NAD⁺-ICDH (Figure 2). They are therefore truncated at the 5' end, within the presumptive mitochondrial import sequence. Assignment of the N-terminus of the mature α -subunit is based on the known pig N-terminal sequence and the high level of sequence conservation between pig, bovine and monkey sequences (Figure 3).

Comparison of mammalian, yeast and bacterial ICDH sequences

The only isocitrate dehydrogenase for which structural information is available is that from *E. coli* [23]. This enzyme is active as a homodimer, and while each subunit contains a separate Mg^{2+} -isocitrate-binding site, isocitrate binding involves residues from the other subunit. Figure 4 compares the sequences of *E. coli* NADP⁺-ICDH and NAD⁺-ICDHs from yeast and mammalian mitochondria. The large insertions present in the *E. coli* sequence are generally surface loops. The sequences of all the subunits of these three enzymes are similar (α and β 43% identity, α and γ 43%, β and γ 51%), implying similarities in their tertiary structures. If the mammalian subunits are compared individually with those of the other enzymes it is apparent that the α -subunit exhibits marginally more similarity to subunit 2 of yeast NAD⁺-ICDH and to *E. coli* NADP⁺-ICDH (Figure 4). Residues which are important in substrate binding and catalysis in the *E. coli* enzyme are indicated in Figure 4, and tabulated together with the equivalent residues from the yeast and mammalian enzymes in Figure 5. Several of these residues (see Figure 5) are not present in the β - or γ -subunits of mammalian NAD⁺-ICDH or subunit 1 of yeast NAD⁺-ICDH. This is consistent with two other observations. Independent mutagenesis of a serine residue important in isocitrate binding (Ser-113 in *E. coli* NADP⁺-ICDH) in both subunits of yeast NAD⁺-ICDH changes the kinetic properties of the enzyme in a way which is consistent with subunit 1 being involved in regulation and subunit 2 in catalysis [26]. Experiments based on separating the α -, β - and γ -subunits of pig NAD⁺-ICDH under minimally denaturing conditions have shown that the α -subunit has more catalytic activity than the other two subunits [27]. This is consistent with mammalian NAD⁺-ICDH α -subunit being responsible for catalytic activity.

In the presence of Mg^{2+} or Mn^{2+} there are two metal ion-isocitrate binding sites per $\alpha_2\beta\gamma$ tetramer of NAD^+ -ICDH, as determined by equilibrium dialysis [7]. In the absence of the bivalent metal ions, four isocitrate molecules bind per tetramer, though with much reduced affinity [28]. As the physiological substrate of the enzyme is Mg^{2+} -isocitrate, it is reasonable to postulate two regulatory and two catalytic isocitrate binding sites per tetramer. Two affinity labels have been used to explore isocitrate binding to NAD^+ -ICDH: 3-bromo-2-oxoglutarate [29] and 3,4-didehydro-2-oxoglutarate [30]. Both are incorporated into all subunits, at a ratio of 1 mol label:1 mol subunit. The affinity labels are also taken up by all subunits individually after purification.

Mammalian NAD⁺-ICDH is regulated by changes in ADP/ATP ratios. Yeast NAD⁺-ICDH is not sensitive to ATP, but is activated by AMP and ADP [7,10]. These differences in the

Figure 4 Alignment of the α - and β -subunits of monkey NAD⁺-ICDH with the γ -subunit [15], both subunits of yeast NAD⁺-ICDH [17,18] and *E. coli* NADP⁺-ICDH [22]

Residues identical in three or more of the six sequences are shaded. Residues important in substrate binding in the *E. coli* enzyme are highlighted with a continuous box, and numbered. Such residues marked ● bind to Mg²⁺-isocitrate, and those marked ◆ bind to NADP. These symbols are accompanied by an asterisk (*) if they are from a separate half of the homodimer. P-loop type adenine nucleotide binding motifs, consensus sequence A/G-X-X-X-G-K-S/T [31], in the mammalian α - and γ -subunits are highlighted by a dotted box.

effects of adenine nucleotides on activity are reflected by the presence in the α - and γ -subunits of the mammalian enzyme of potential adenine nucleotide binding sites [31] (Figure 4), which are not present in either subunit of yeast NAD⁺-ICDH. While there is some evidence for two ADP-binding sites per $\alpha_2\beta_2\gamma$ tetramer of mammalian NAD⁺-ICDH [5], it is not known whether ATP and ADP compete for the same binding site.

The most striking difference between NAD⁺-ICDHs from mammalian and yeast mitochondria is that only the mammalian enzyme is activated by Ca²⁺. There is no significant similarity

between any of the subunits of the mammalian enzyme and known Ca²⁺-binding proteins. Furthermore, the 38–45% identity between the deduced amino acid sequences of the yeast and mammalian enzymes extends throughout all three subunits of mammalian NAD⁺-ICDH (Figure 4). There is no obvious domain where the sequence of one of the mammalian subunits is different from either of the yeast subunits. Thus, although the availability of cDNA clones for all three subunits of mammalian NAD⁺-ICDH sheds some light on the distribution of regulatory and catalytic roles among individual subunits, there is still no

<i>E. coli</i> NADP ⁺ -ICDH	Yeast NAD ⁺ -ICDH1	Yeast NAD ⁺ -ICDH2	Monkey NAD ⁺ -ICDH α	Monkey NAD ⁺ -ICDH β	Monkey NAD ⁺ -ICDH γ
S-113	S-92	S-99	S-91	S-93	S-91
N-115	N-94	N-101	N-93	N-95	N-93
R-119	R-98	R-105	R-97	R-99	R-97
R-129	R-109	R-115	R-107	R-109	R-109
R-153	R-128	R-136	R-128	R-130	R-128
Y-160	Y-125	Y-143	Y-135	Y-137	Y-135
K-230*	K-182	K-190	K-182	K-184	K-182
D-283*	D-216	D-223	D-215	D-217	D-215
D-307	D-239	D-249	D-239	D-241	D-239
D-311	D-243	D-253	D-243	D-245	D-243

Figure 5 Conservation of residues which bind to isocitrate in *E. coli* NADP⁺-ICDH, in yeast and monkey NAD⁺-ICDHs

Crystallographic studies have identified residues involved in isocitrate binding in *E. coli* NADP⁺-ICDH [23]. Such residues are tabulated together with those in equivalent positions in the alignment of yeast and monkey NAD⁺-ICDHs in Figure 3. Non-conserved residues are shaded. A full complement of isocitrate binding residues is present in the α -subunit of the monkey enzyme and subunit 2 of yeast NAD⁺-ICDH. Residues in the *E. coli* enzyme which are from a separate half of the homodimer are indicated with an asterisk (*). The single-letter amino acid code is used.

evidence as to the molecular basis for the regulation of this enzyme by Ca²⁺. Either the NAD⁺-ICDH Ca²⁺-binding site is novel, or Ca²⁺ binds to a previously undetected, low-molecular-mass, Ca²⁺-binding subunit.

Since this paper was originally submitted, the sequence of the α -subunit of human NAD⁺-ICDH has been published [32]. The deduced amino acid sequence of the human and monkey α -subunits differ at two residues.

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